Acta Biomaterialia xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Genetically engineered silk–collagen-like copolymer for biomedical applications: Production, characterization and evaluation of cellular response

Małgorzata K. Włodarczyk-Biegun [a], Marc W.T. Werten [b], Frits A. de Wolf [b], Jeroen J.J.P. van den Beucken [c], Sander C.G. Leeuwenburgh [c], Marleen Kamperman [a,*], Martien A. Cohen Stuart [a]

[a] Laboratory of Physical Chemistry and Colloid Science, Wageningen University, Dreijenplein 6, NL-6703 HB Wageningen, The Netherlands
[b] Wageningen UR Food & Biobased Research, Bornse Weilanden 9, NL-6708 WG Wageningen, The Netherlands
[c] Department of Biomaterials, Radboud University Medical Centre, Philips van Leydenlaan 25, NL-6525 EX Nijmegen, The Netherlands

ARTICLE INFO

Article history:
Received 20 December 2013
Received in revised form 24 March 2014
Accepted 2 May 2014
Available online xxxx

Keywords:
Genetically engineered protein polymers
Silk–collagen-like block copolymers
Hydrogels
Pichia pastoris
Cellular response

ABSTRACT

Genetically engineered protein polymers (GEPP) are a class of multifunctional materials with precisely controlled molecular structure and property profile. Representing a promising alternative for currently used materials in biomedical applications, GEPP offer multiple benefits over natural and chemically synthesized polymers. However, producing them in sufficient quantities for preclinical research remains challenging. Here, we present results from an in vitro cellular response study of a recombinant protein polymer that is soluble at low pH but self-organizes into supramolecular fibers and physical hydrogels at neutral pH. It has a triblock structure denoted as $C_2S^H_{48}C_2$, which consists of hydrophilic collagen-inspired and histidine-rich silk-inspired blocks. The protein was successfully produced by the yeast *Pichia pastoris* in laboratory-scale bioreactors, and it was purified by selective precipitation. This efficient and inexpensive production method provided material of sufficient quantities, purity and sterility for cell culture study. Rheology and erosion studies showed that it forms hydrogels exhibiting long-term stability, self-healing behavior and tunable mechanical properties. Primary rat bone marrow cells cultured in direct contact with these hydrogels remained fully viable; however, proliferation and mineralization were relatively low compared to collagen hydrogel controls, probably because of the absence of cell-adhesive motifs. As biofunctional factors can be readily incorporated to improve material performance, our approach provides a promising route towards biomedical applications.

1. Introduction

Genetically engineered protein (block co)polymers (GEPP), with blocks inspired by animal extracellular matrix proteins, have great potential in modern medicine [1,2]. Since the pioneering work of Doel, Capello, Tirrell and others [3], several well-defined polymers, composed of collagen-like [4,5], elastin-like [6–8] or silk-like amino acid blocks [9–12], have been produced using recombinant DNA technology [1,13]. This material class offers several benefits over synthetic polymers and animal-derived biopolymers [1,2,14,15].

First of all, structural elements found in nature, such as (triple) helices, β-sheets and β-rolls, can be combined in GEPP with great flexibility, enabling control over structure and material properties. Second, a desired number of biofunctional domains, such as cell binding sites, can be incorporated into the protein sequence. These benefits allow the design of biomimetic scaffolds that resemble the structure and composition of native extracellular matrix, and can induce desired cell responses [1,16,17]. Third, there is little batch-to-batch variation in GEPP. Once the genetically modified host organism for production has been constructed, the material production process, consisting of fermentation and purification, can be routinely repeated. Fourth, the recombinant product (and all its constituting blocks) is monodisperse as the biosynthetic pathway ensures that all individual molecules are, in principle, identical. Finally, common concerns with animal derivatives, such as the risk of uncontrolled degradation and possible contamination with transmissible disease agents, are avoided [1,17].

Although the field of GEPP offers many possibilities, some critical challenges remain. One of the main limitations for the use of GEPP in preclinical materials research is the low product

* Corresponding author. Tel.: +31 317 482 178; fax: +31 317 483 777.
E-mail address: marleen.kamperman@wur.nl (M. Kamperman).

http://dx.doi.org/10.1016/j.actbio.2014.05.006
1742-7061/

yield and the resulting small quantities of material that are typically obtained in the laboratory [1]. Our group was the first to report the use of the methylotrophic yeast *Pichia pastoris* for the secreted production of GEPP with a repetitive sequence [4], and has since successfully produced recombinant collagen-, silk- and elastin-like protein polymers at relatively high yields [18–23]. This includes the silk–collagen-like triblock copolymer protein [21,24] used in this work, which is produced at grams per liter levels [21]. In addition to high yields, the advantages of the *P. pastoris* system include the ability to produce highly repetitive sequences [13] and to perform most post-translational modifications [25]. The presently used protein can self-assemble into a nanofibrous hydrogel and has structural and functional properties similar to those of the extracellular matrix of connective tissue. The polymer, denoted further as $\boldsymbol{C_2S^H_{48}C_2}$, consists of a middle block ($\boldsymbol{S^H_{48}}$) composed of 48 identical silk-like octapeptides in tandem, and two end blocks ($\boldsymbol{C_2}$), each of which are composed of two identical 99 amino acid-long collagen-like polypeptides in tandem [21]. Like in natural collagen, glycine occurs as every third amino acid, and the incidence of proline residues is ~22% in the $\boldsymbol{C_2}$ modules. Proline residues suppress β-sheet or α-helix formation, yet promote triple helix formation. However, the proline content in the $\boldsymbol{C_2}$ modules is too low to induce triple helix formation without post-translational hydroxylation. Therefore, because all the other amino acids in $\boldsymbol{C_2}$ are hydrophilic (mainly uncharged), and because the proline residues are not post-translationally hydroxylated, $\boldsymbol{C_2}$ assumes a random coil conformation, irrespective of temperature and pH [19]. The silk-like repeating unit in the middle block $\boldsymbol{S^H_{48}}$ is (in one-letter amino acid notation) GAGAGAGH. At low pH the histidine residues in the $\boldsymbol{S^H_{48}}$ domain are charged and hence the block is unfolded and water soluble. Upon increasing the pH to the condition of body fluid (pH 7.4), the charge on the $\boldsymbol{S^H_{48}}$ domain is reduced and the repulsion between the chains decreases, which enables $\boldsymbol{S^H_{48}}$ to fold and form supramolecular stacks. The conformational transition of $\boldsymbol{S^H_{48}}$, in combination with colloidal stabilization by the hydrophilic, randomly coiling $\boldsymbol{C_2}$ blocks in $\boldsymbol{C_2S^H_{48}C_2}$, results in the formation of long nanofibers, making dilute fiber networks (gels) with an open structure at physiological pH. Since these gels are formed by physical forces, no addition of external crosslinkers is needed, which avoids the risk of the formation of toxic by-products.

Here, we present a thorough characterization of the properties of $\boldsymbol{C_2S^H_{48}C_2}$ hydrogels that are relevant for cell culture and tissue engineering. The characterization included rheological and erosion analysis, as well as viability, proliferation and mineralization tests after culturing primary rat bone marrow stem cells (MSC) in direct contact with these hydrogels.

2. Materials and methods

2.1. Fermentation

The development of a *P. pastoris* strain for $\boldsymbol{C_2S^H_{48}C_2}$ protein production was described recently by our group [21]. The $\boldsymbol{C_2S^H_{48}C_2}$ protein was produced by methanol-fed batch fermentation in 3 and 7 l Bioflo bioreactors (New Brunswick Scientific) at 30 °C and pH 3, as described previously [21]. The microfiltered, cell-free supernatant was stored at −20 °C prior to use.

2.2. Purification of $\boldsymbol{C_2S^H_{48}C_2}$ protein

The $\boldsymbol{C_2S^H_{48}C_2}$ proteins were purified following a procedure that was modified from the previously reported procedure [21] to ensure high purity. The supernatant (450 ml) was thawed in a water bath at 25 °C. Next, the $\boldsymbol{C_2S^H_{48}C_2}$ protein was selectively precipitated from the supernatant by adding ammonium sulphate over the course of 30 min to a final concentration amounting to 45% of saturation under continuous stirring, followed by mild stirring at 4 °C for 1 h and centrifugation at 16,000 *g* at 4 °C in a Sorvall SLA-1500 rotor for 30 min. The obtained pellet was dissolved overnight in 450 ml of 50 mM formic acid under continuous stirring at 4 °C, after which the precipitation procedure was repeated. The obtained pellet was redissolved overnight in 150 ml of 50 mM formic acid under continuous stirring at 4 °C and the $\boldsymbol{C_2S^H_{48}C_2}$ protein was precipitated by the addition of acetone to a final concentration of 80% (v/v). The obtained pellet was redissolved overnight in 225 ml of 50 mM formic acid under continuous stirring at 4 °C and dialyzed (Spectrum Labs Spectra/Por 7 dialysis tubing with 1 kDa nominal molecular weight cut-off), first against 50 mM formic acid at 4 °C for 24 h (three buffer replacements) and then against 10 mM formic acid at 4 °C for 16 h (two buffer replacements). The final product was microfiltered (membrane pore size 0.2 μm) to sterilize the material and to remove any protein aggregates. After microfiltration, the protein was freeze-dried and kept under sterile conditions. It should be noted that freeze-drying was not performed under sterile conditions and no additional sterilization treatment was performed before the cell culture studies.

2.3. Material characterization

2.3.1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein purity was assessed by SDS–PAGE using the Invitrogen NuPAGE Novex system, with 10% (w/v) Bis–Tris gel, MES SDS running buffer and SeeBlue Plus2 molecular mass marker. Gels were stained using Coomassie SimplyBlue SafeStain.

2.3.2. N-terminal sequencing

N-terminal sequencing by Edman degradation was performed for the SDS–PAGE gel band corresponding to apparent mass of ~62 kDa (indicated by an arrow in Fig. 1). Proteins were blotted onto a 0.2 μm polyvinylidene difluoride membrane (Invitrogen) using CAPS buffer (10 mM N-cyclohexyl-3-aminopropanesulfonic acid, pH 11, 10% (v/v) ethanol). After staining with Coomassie SimplyBlue SafeStain, selected band was cut out and sent for sequencing. Protein sequencing was performed by Midwest Analytical (St. Louis, MO, USA).

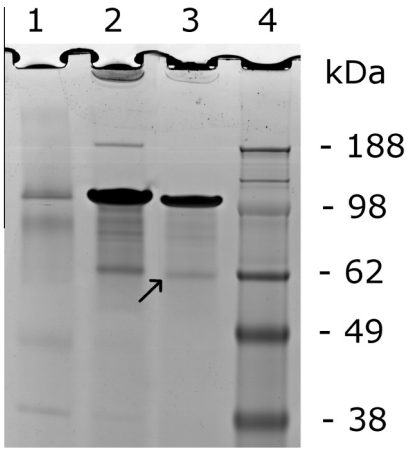

Fig. 1. SDS–PAGE of $\boldsymbol{C_2S^H_{48}C_2}$ protein after consecutive purification steps: lane 1: cell free fermentation broth; lane 2: redissolved pellet after the first ammonium sulphate precipitation; lane 3: final product after dialysis and freeze-drying; lane 4: molecular weight marker. The arrow is pointing at the band extracted for N-terminal sequencing. Note that the precipitated proteins were concentrated and band intensities cannot be compared.